

# Serotonin (5-HT) Receptor Subtypes Mediate Specific Modes of 5-HT-Induced Signaling and Regulation of Neurosecretion in Gonadotropin-Releasing Hormone Neurons

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Serotonin (5-HT), the endogenous nonselective 5-HT receptor agonist, activates the inositol 1,4,5-triphosphate/calcium ( $\text{InsP}_3/\text{Ca}^{2+}$ ) signaling pathway and exerts both stimulatory and inhibitory actions on cAMP production and GnRH release in immortalized GnRH neurons. The high degree of similarity between the signaling and secretory responses elicited by GnRH and 5-HT prompted us to target specific 5-HT receptor subtypes to deconvolute the complex actions of these agonists on signal transduction and GnRH release. Specific mRNA transcripts for 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>4</sub>, and 5-HT<sub>7</sub> were identified in immortalized GnRH neurons (GT1–7). The rate of firing of spontaneous action potentials (APs) by hypothalamic GnRH neurons and cAMP production and pulsatile GnRH release in GT17 cells were profoundly inhibited during activation of the  $\text{G}_i$ -coupled 5-HT<sub>1A</sub> receptor. Treatment with a selective agonist to activate the  $\text{G}_q$ -coupled 5-HT<sub>2C</sub> receptor increased the rate of

firing of spontaneous APs, stimulated  $\text{InsP}_3$  production and caused a delayed increase in GnRH release. Selective activation of the  $\text{G}_s$ -coupled 5-HT<sub>4</sub> receptor also increased the rate of firing of APs, stimulated cAMP production, and caused a sustained and robust increase in GnRH release. The ability of 5-HT receptor subtypes expressed in GnRH neurons to activate single or multiple G proteins in a time- and dose-dependent manner differentially regulates the phospholipase C/ $\text{InsP}_3/\text{Ca}^{2+}$ , and adenylyl cyclase/cAMP signaling pathways, and thereby regulates the frequency and amplitude of pulsatile GnRH release. This process, in conjunction with the modulation of spontaneous electrical activity of the GnRH neuron, contributes to the control of the pulsatile mode of neuropeptide secretion that is characteristic of GnRH neuronal function *in vivo* and *in vitro*. (*Molecular Endocrinology* 20: 125–135, 2006)

**P**ULSATILE RELEASE OF GnRH into the hypothalamic/pituitary portal vessels at the median eminence is essential for the maintenance of optimal gonadotropin secretion and normal reproductive function. Several mechanisms for the generation of pulsatile GnRH release have been suggested, including the spontaneous electrical activity of single GnRH

neurons (1, 2), bursts of action potentials as in other neuroendocrine cells (3–6), and cAMP-dependent activation of cyclic nucleotide-gated cation channels in normal and immortalized GnRH neurons (7–10). GnRH action in GT1–7 neurons is also associated with activation of phospholipase D. This response is mediated by PKC and  $\text{Ca}^{2+}$  influx through voltage-sensitive calcium channels, and phospholipase D serves as a common intracellular effector for phospholipase C (PLC)- and voltage-gated signaling pathways in GnRH neurons (11).

The secretory activities of GnRH neurons *in vivo* and *in vitro* are influenced by neurotransmitters (12), catecholamines (13), opiates (14), neuropeptides (15, 16), pituitary hormones (17, 18), and gonadal steroids (19, 20). In addition, the expression of mRNAs for GnRH and GnRH receptor, and the modulation of pulsatile GnRH release by GnRH agonist and antagonist analogs, indicates that an autocrine GnRH regulatory system is operative in native and immortalized GnRH neurons (21, 22). Within the hypothalamus, the autoregulatory control of GnRH neuronal activity is integrated with other neuronal and hormonal inputs to

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Abbreviations: AC, Adenylyl cyclase; AP, action potential; E-18, embryonic d 18; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; 5-HT, serotonin;  $\text{InsP}_{2+3}$ , inositol biphosphate + inositol triphosphate;  $\text{InsP}_3$ , inositol 1,4,5-triphosphate;  $\alpha$ -methyl 5-HT,  $\alpha$ -methyl-5-hydroxytryptamine; 2-MPP, 1-(2-methoxyphenyl)piperazine; NK, neurokinin; nt, nucleotide; PAPP, 4-[2-[4-[3-(trifluoromethyl)phenyl]-1-piperazinyl]ethyl]benzeneamine *p*-aminophenethyl-*m*-trifluoromethylphenyl piperazine; PLC, phospholipase C; PTX, pertussis toxin.

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provide a more complex control system with a high degree of redundancy to drive and maintain pulsatile GnRH release and reproductive function.

The serotonergic system is but one of several neuronal inputs that innervate hypothalamic GnRH neurons (23). The demonstration of synaptic contacts between tritiated 5-HT-labeled buttons and GnRH-immunoreactive neurons by Kiss and Halasz (23) suggested that 5-HT-containing neurons could act directly on GnRH release. In animal studies, 5-HT exerts both stimulatory and inhibitory effects on GnRH release, depending on age, gender, and the signaling pathways of the individual 5-HT receptor subtypes (12, 24–26).

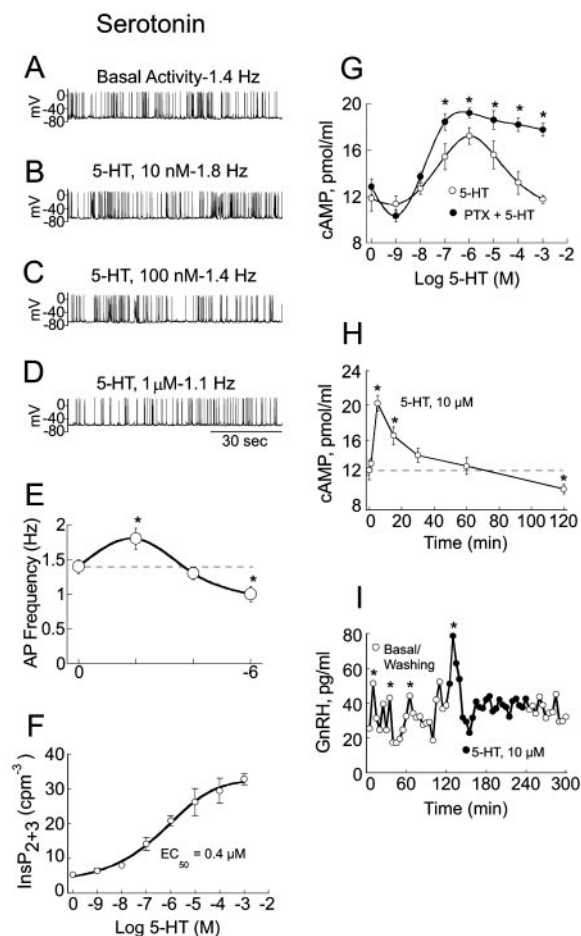
The five subtypes of G protein-coupled 5-HT receptors are differentially coupled to at least three specific G proteins (5-HT<sub>1</sub>, primarily to G<sub>i</sub>; 5-HT<sub>2</sub>, primarily to G<sub>q</sub>; 5-HT<sub>4</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub>, primarily to G<sub>s</sub>), and have been classified by structural and transductional criteria. Their two major intracellular second messenger pathways are mediated by regulation of adenylyl cyclase (AC) and PLC and phospholipase D. In the present studies, the effects of 5-HT receptor activation on membrane excitability, intracellular signaling, and GnRH secretion were analyzed in native hypothalamic GnRH neurons and immortalized GnRH neurons (GT1–7 neurons), which have similar cellular and functional properties (21, 27). Identified hypothalamic GnRH neurons were used for electrophysiological recordings, and receptor expression, cellular signaling, and GnRH secretion were analyzed in cultured GT1–7 neurons. Changes in membrane excitability, intracellular signaling, and neuropeptide secretion were examined during treatment with selective agonist and antagonist analogs to elucidate the roles of individual 5-HT receptor subtypes in the complex regulation of pulsatile GnRH release.

## RESULTS

### 5-HT-Induced Changes in Electrical Activity of Native GnRH Neurons and Signaling Pathways and Secretory Responses

5-HT, the endogenous nonselective 5-HT receptor agonist, caused biphasic and dose-dependent changes in the firing of action potentials (APs) in hypothalamic GnRH neurons. AP firing significantly increased during treatment with 10 nM 5-HT ( $1.4 \pm 0.1$  Hz to  $1.9 \pm 0.13$  Hz;  $P < 0.01$ ;  $n = 5$ ; Fig. 1, B and E), gradually decreased to the basal rate of firing during treatment with 100 nM 5-HT (Fig. 1, C and E), and was significantly reduced during treatment with 1  $\mu$ M 5-HT ( $1.4 \pm 0.1$  Hz to  $1.0 \pm 0.05$  Hz;  $P < 0.05$ ;  $n = 5$ ; Fig. 1, D and E).

In GT1–7 neurons, treatment with 5-HT activated the inositol 1,4,5-triphosphate (InsP<sub>3</sub>)/Ca<sup>2+</sup> signaling pathway and caused a monophasic, dose-dependent increase in inositol bisphosphate + inositol trisphosphate (InsP<sub>2+3</sub>) production (Fig. 1F). In addition to



**Fig. 1.** 5-HT-Induced Changes in Electrical Activity, Signaling Pathways, and Pulsatile Neurosecretion in Native and Immortalized GnRH Neurons

A, Spontaneous firing of APs in identified E-18 hypothalamic GnRH neurons. B, Stimulation of AP firing by low nanomolar 5-HT concentrations. C, Lack of changes in AP firing during treatment with high nanomolar 5-HT concentrations. D, Inhibition of AP firing by micromolar 5-HT concentrations. E, Dose-dependent effect of 5-HT on AP firing in identified GnRH neurons. All AP traces were obtained from identified hypothalamic GnRH neurons. F, Dose-dependent stimulation of InsP<sub>2+3</sub> production in GT1–7 neurons. G, Bell-shaped dose-dependent effects of 5-HT on cAMP production in GT1–7 neurons (open circles). Reversal of 5-HT-induced inhibition of cAMP production by pertussis toxin (solid circles). H, Time-dependent action of 5-HT on cAMP production in GT1–7 neurons. Data are means  $\pm$  SEM of four independent experiments. I, Initial stimulation and subsequent inhibition of pulsatile GnRH release during treatment with 5-HT. A representative profile from three independent perfusion experiments is shown in this and subsequent figures. (M), Molar concentration.

stimulating the inositol phosphate/Ca<sup>2+</sup>-signaling pathway via G<sub>q/11</sub>, 5-HT also regulates cAMP production. The action of 5-HT on cAMP production in GT1–7 neurons was also biphasic and dose dependent. 5-HT concentrations of up to 1  $\mu$ M caused a progressive increase in cAMP production, but at higher concen-

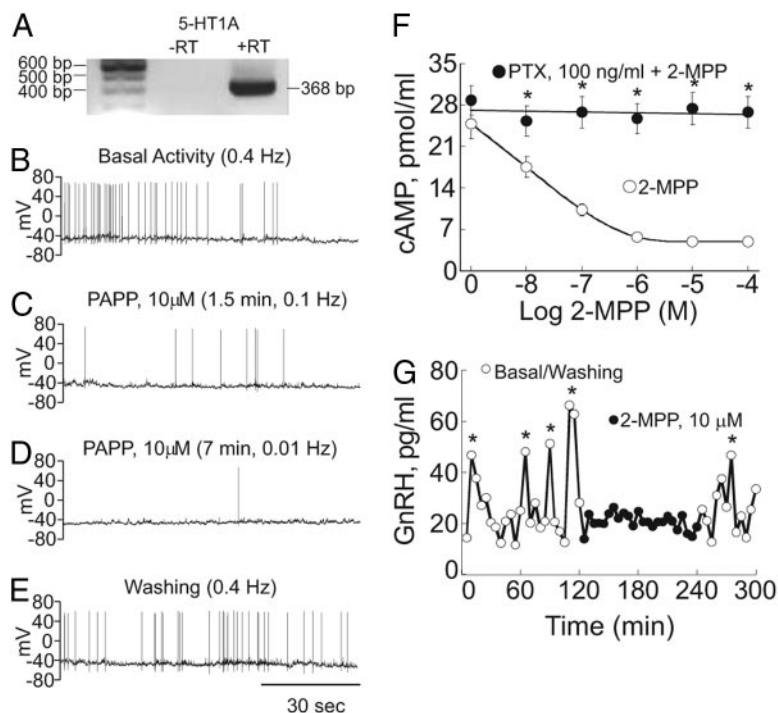
trations 5-HT had an inhibitory effect on cAMP production that was prevented by treatment with pertussis toxin (PTX). The bell-shaped dose-response curve and the reversal of the inhibitory actions of high 5-HT concentrations on cAMP production by PTX are consistent with the activation of  $G_i$ -related proteins (Fig. 1G). In time studies, 5-HT treatment elicited a biphasic response in cAMP production. During sustained treatment, an initial increase during the first 30 min was followed by a reduction in cAMP production to below the basal level after 60 min (Fig. 1H). The  $\text{InsP}_{2+3}$  and cAMP responses were differentially regulated by increasing agonist concentrations, such that  $\text{IP}_{2+3}$  production was maximal at high 5-HT concentrations, when cAMP production was minimal. In contrast, maximal cAMP production occurred during stimulation with low 5-HT concentrations, at which  $\text{IP}_{2+3}$  production was half-maximal (Fig. 1, F and G).

These time- and dose-dependent differences in the activation of specific second messengers were also reflected in the profile of GnRH release. In perfused GT1–7 neurons, the GnRH secretory profile was characterized by clearly defined peaks with mean ampli-

tude of  $35.4 \pm 3.8$  pg/ml ( $n = 3$ ) and interpeak intervals of  $39.4 \pm 5.3$  min. Application of  $10 \mu\text{M}$  5-HT caused a transient increase in GnRH peak amplitude (to  $49.3 \pm 4.5$  pg/ml;  $n = 3$ ;  $P < 0.05$ ) followed by cessation of pulsatile GnRH release (Fig. 1I).

### Expression of $G_i$ -Coupled 5-HT<sub>1A</sub> Receptors and Their Effects on Electrical Activity, Second Messengers, and Secretory Responses

Analysis of total RNA from cultured GT1–7 neurons, using gene-specific primers based on the 5-HT<sub>1A</sub> receptor sequence, gave the expected size fragment of 368 bp. No such products were obtained in the absence of reverse transcribed mRNA, indicating that the RNA preparation was free of genomic DNA contamination. Real-time quantitative RT-PCR revealed  $7.4 \pm 0.8 \times 10^4$  ( $n = 3$ ) copies of 5-HT<sub>1A</sub> receptor/ $\mu\text{g}$  DNA (Fig. 2A). DNA sequencing of the purified band confirmed the authenticity of the amplified fragment, because the nucleotide sequences matched the published sequences of the 5-HT<sub>1</sub> receptor (data not shown).



**Fig. 2.** Expression of 5-HT<sub>1A</sub> Receptor Transcripts and Ligand-Induced Changes in Electrical Activity, Second Messengers, and Pulsatile Neurosecretion in Native and Immortalized GnRH Neurons

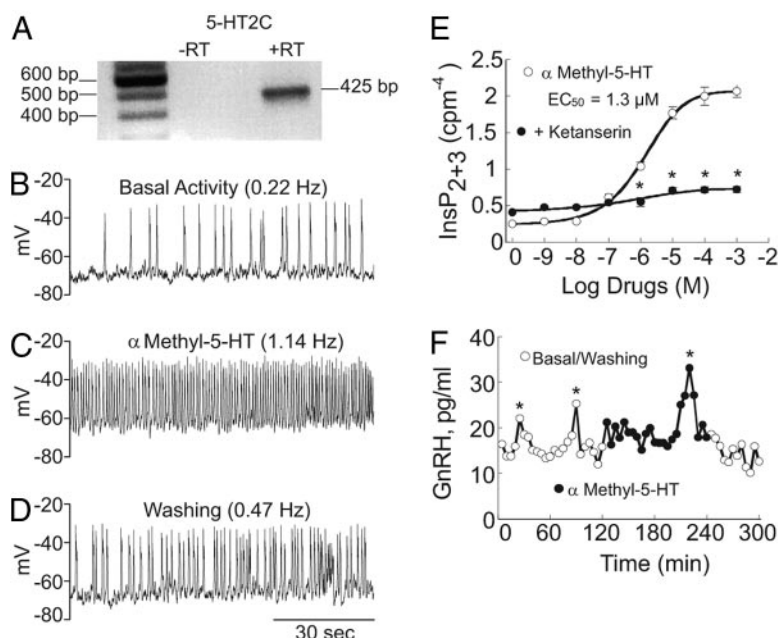
A, Expression of 5-HT<sub>1A</sub> receptors in cultured GT1–7 neurons was demonstrated using gene-specific primers and was confirmed by DNA sequencing of the purified band. B, Spontaneous firing of APs in identified cultured E-18 hypothalamic GnRH neurons. C and D, Time-dependent inhibition of AP firing in native GnRH neurons during treatment with PAPP, a 5-HT<sub>1A</sub> receptor agonist. E, Resumption of spontaneous AP firing during washout of PAPP. All AP traces were obtained from identified hypothalamic GnRH neurons. F, Dose-dependent inhibition of cAMP production during treatment with 2-MPP, another 5-HT<sub>1A</sub> receptor agonist. The data are means from four independent experiments. G, Inhibition of pulsatile GnRH secretion during treatment with 2-MPP (solid circles), and resumption of pulsatile GnRH release during the washout period. (M), Molar concentration; RT, reverse transcription.

The basal rate of AP firing in hypothalamic GnRH neurons (Fig. 2B) was markedly reduced during treatment with the selective 5-HT<sub>1A</sub> receptor agonist, 4-[2-[4-[3-(trifluoromethyl)phenyl]-1-piperazinyl]ethyl]-benzeneamine *p*-aminophenethyl-*m*-trifluoromethyl-phenyl piperazine (PAPP) (10  $\mu$ M), from  $0.4 \pm 0.05$  Hz to  $0.1 \pm 0.03$  Hz ( $P < 0.01$ ;  $n = 8$ ) after 1.5 min of treatment and was almost completely abolished after 7 min (Fig. 2D). Spontaneous AP firing resumed during washout of the agonist and returned to the control level (Fig. 2E). Treatment of GT1–7 neurons with 1-(2-methoxyphenyl)piperazine (2-MPP), another potent and selective agonist for G<sub>i/o</sub>-coupled 5-HT<sub>1A</sub> receptors, inhibited cAMP production in a dose-dependent manner (*open circles*). This inhibitory response to 2-MPP was prevented by treatment with PTX, consistent with its activation of G<sub>i</sub>-related proteins (Fig. 2F, *solid circles*). Pulsatile GnRH release was also markedly inhibited during selective activation of the G<sub>i</sub>-coupled 5HT<sub>1A</sub> receptor with 2-MPP (Fig. 2G). The characteristic pulsatile GnRH release from perfused GT1–7 neurons was completely abolished, and the mean basal GnRH release decreased from  $28.3 \pm 0.3$  pg/ml to  $18.4 \pm 0.3$  pg/ml in cultures perfused with

100  $\mu$ M 2-MPP. Pulsatile GnRH release subsequently resumed during the washout period.

### Expression of G<sub>i</sub>-Coupled 5-HT<sub>2C</sub> receptors and Their Effects on Electrical Activity, Second Messengers, and Secretory Responses

The expression of 5-HT<sub>2C</sub> receptors in cultured GT1–7 neurons was demonstrated by real-time RT-PCR. Analysis of total RNA, using gene-specific primers based on sequences of the 5-HT<sub>2C</sub> receptor, gave the expected fragment size of 452 bp. No such products were obtained in the absence of reverse-transcribed mRNA, indicating that the RNA preparation was free of genomic DNA contamination. Real-time PCR was also used to quantify transcript levels of the individual 5-HT receptors in cultured GT1–7 neurons. Based on the number of copies present in the cDNA relative to the standard curve, cultured GT1–7 neurons expressed  $3.1 \pm 0.4 \times 10^5$  ( $n = 3$ ) copies of 5-HT<sub>2C</sub> receptor/ $\mu$ g DNA. The expression of 5-HT<sub>2C</sub> receptor genes in cultured GT1–7 neurons and the effects of their activation on neuronal firing are shown in Fig. 3A. DNA sequencing of the purified band confirmed the



**Fig. 3.** Expression of 5-HT<sub>2C</sub> Receptor Transcripts and Ligand-Induced Changes in Electrical Activity, Second Messengers, and Pulsatile Neurosecretion in Native and Immortalized GnRH Neurons

A, Expression of 5-HT<sub>2C</sub> receptors in cultured GT1–7 neurons as demonstrated with gene-specific primers. DNA sequencing of the purified band confirmed the authenticity of the amplified fragment and matched the published sequence of the 5-HT<sub>2C</sub> receptor. B, Spontaneous firing of APs in identified cultured E-18 hypothalamic GnRH neurons. Recordings were obtained from single isolated GnRH neurons to eliminate the influences of electrical and synaptic coupling between cells. C, Increased frequency of AP firing in native GnRH neurons treated with  $\alpha$ -methyl-5-HT. D, Reduction of spontaneous AP firing during washout of the 5-HT<sub>2C</sub> receptor agonist. All traces were obtained from identified hypothalamic GnRH neurons. E, Dose-dependent stimulation of IP<sub>2+3</sub> production during treatment with a 5-HT<sub>2C</sub> receptor agonist (*open circles*). Prevention of the stimulatory action of the 5-HT<sub>2C</sub> receptor agonist on IP<sub>2+3</sub> production in the presence of the selective agonist (*solid circles*). Data are the means from three independent experiments. F, Prominent increase in GnRH peak amplitude during treatment with a 5-HT<sub>2C</sub> receptor agonist (*solid circles*), and resumption of basal pulsatile GnRH release during the washout period. (M), Molar concentration; RT, reverse transcription.

authenticity of the amplified fragment, which matched the published sequence of the 5-HT<sub>2C</sub> receptor (data not shown).

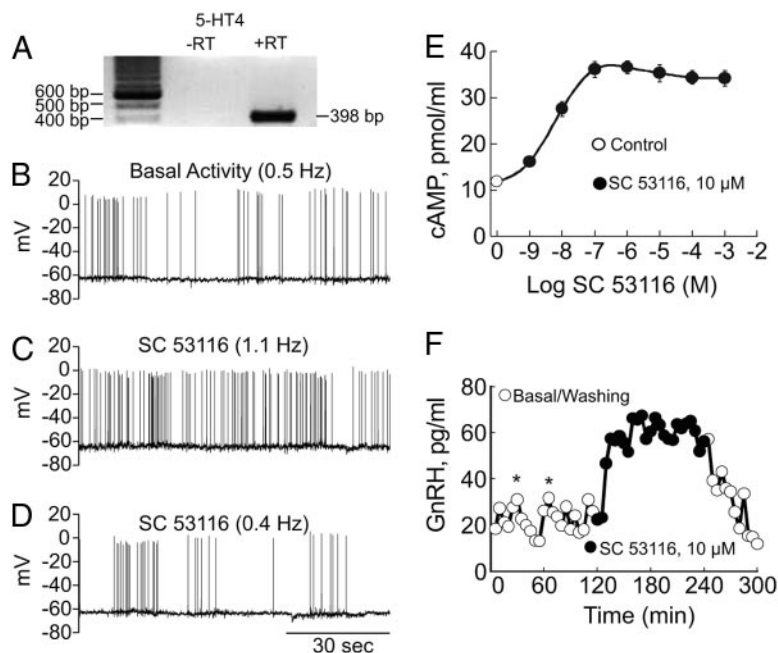
Whole-cell recordings from native GnRH neurons consistently exhibited spontaneous AP firing, and most of the cells (70%) showed irregular spiking activity (Fig. 3B). Treatment with 10  $\mu$ M  $\alpha$ -methyl-5-hydroxytryptamine ( $\alpha$ -methyl 5-HT), a selective agonist for the G<sub>s</sub>-coupled 5-HT<sub>2</sub> receptor, significantly increased the frequency of AP firing, from basal ( $0.22 \pm 0.05$  Hz to  $1.14 \pm 0.9$  Hz;  $P < 0.01$ ;  $n = 10$ ) (Fig. 3C). During washout of the agonist, spontaneous AP firing returned to the control level after 3 min (Fig. 3D).

Treatment of GT1–7 neurons with  $\alpha$ -methyl 5-HT stimulated InsP<sub>2+3</sub> productions in a dose-dependent manner, with EC<sub>50</sub> of 1.3  $\mu$ M and a maximal response at micromolar agonist concentrations (Fig. 3E). Treatment with 10  $\mu$ M  $\alpha$ -methyl 5-HT significantly increased GnRH peak amplitude after an initial delay ( $21.3 \pm 2.2$  pg/ml control vs.  $32.3 \pm 3.5$  pg/ml treated;  $n = 3$ ;  $P < 0.05$ ; Fig. 3F). This response is consistent with earlier observations that activation of the PLC/InsP<sub>3</sub>/Ca<sup>2+</sup> signaling pathway increases GnRH pulse amplitude and reduces pulse frequency (21, 22).

### Expression of G<sub>s</sub>-Coupled 5-HT<sub>4</sub> Receptors and Their Effects on Electrical Activity, Second Messengers, and Secretory Responses in GnRH Neuronal Cells

Analysis of total RNA from cultured GT1–7 neurons, using gene-specific primers based on sequences of the G<sub>s</sub>-coupled 5-HT<sub>4</sub> receptor, gave the expected size fragment of 398 bp (Fig. 4A). No such products were obtained in the absence of reverse transcribed mRNA. Real-time quantitative RT-PCR detected  $5.3 \pm 0.8 \times 10^4$  ( $n = 3$ ) copies of 5-HT<sub>4</sub> receptor/ $\mu$ g DNA. DNA sequencing of the purified band confirmed the authenticity of the amplified fragment, because the nucleotide sequence matched the known sequence of the 5-HT<sub>4</sub> receptor (data not shown). 5-HT<sub>7</sub> receptors were also expressed in GT1–7 neurons at low levels ( $2.1 \times 10^3$  copies/mg DNA).

In native GnRH neurons, the basal rate of AP firing (Fig. 4B) was significantly increased during treatment with 10  $\mu$ M SC 53116, a selective agonist for the G<sub>s</sub>-coupled 5-HT<sub>4</sub> receptor, from basal ( $0.5 \pm 0.05$  Hz to  $1.1 \pm 0.2$  Hz;  $P < 0.01$ ;  $n = 12$ ) (Fig. 4C). Spontaneous AP firing decreased during washout of the agonist and returned to the control level after 3 min (Fig.



**Fig. 4.** Expression of 5-HT<sub>4</sub> Receptor Transcripts and Ligand-Induced Changes in Electrical Activity, Second Messengers, and Pulsatile Neurosecretion in Native and Immortalized GnRH Neurons

A, Expression of 5-HT<sub>4</sub> receptors in cultured GT1–7 neurons was demonstrated using gene-specific primers and was confirmed by DNA sequencing of the purified band. B, Spontaneous firing of APs in identified cultured E-18 hypothalamic GnRH neurons. C, Increased frequency of AP firing in native GnRH neurons treated with the 5-HT<sub>4</sub> receptor agonist, SC 53116. D, Reduction of spontaneous AP firing during washout of the 5-HT<sub>4</sub> receptor agonist. All traces were obtained from identified hypothalamic GnRH neurons. E, Dose-dependent stimulation of cAMP production during treatment with SC 53116. Data are the means from four independent experiments. F, Prolonged and prominent increase in GnRH secretion during treatment with SC 53116 (solid circles), and resumption of basal pulsatile GnRH release during the washout period. (M), Molar concentration; SC 53116, (1*S*,8*S*)-*n*-[(hexahydro-1*H*-pyrrolizin-1-yl)methyl]-4-amino-5-chloro-2-methoxy-benzamide.

4D). Treatment of GT1–7 neurons with SC 531116 caused dose-dependent stimulation of cAMP production, which was maximal at 100 nM SC 53116 and showed no further increase at micromolar concentrations (Fig. 4E). Activation of the 5-HT<sub>4</sub> receptor with SC 53116 also caused a sustained and robust increase in GnRH release, which increased from  $23.2 \pm 3.6$  pg/ml in controls to  $58.4 \pm 6.4$  pg/ml in treated cells ( $P < 0.01$ ;  $n = 3$ ) and returned to the basal level during the washout period (Fig. 4F).

## DISCUSSION

The complexity of GnRH-induced intracellular signaling and its regulation of gene expression, ion channels, enzyme activity, and hormone secretion in native and immortalized GnRH neurons reflects the existence of diverse signaling pathways activated by the single GnRH receptor. In contrast, multipathway signaling induced by other G protein-coupled receptor (GPCR) agonists is often attributable to their effects on diverse receptor subtypes. Recent research has identified additional mechanisms that can increase the diversity of signaling pathways emanating from a single GPCR. These include the existence of multiple endogenous ligands with differential affinities for a particular receptor subtype that activate different responses and the ability of individual receptors to couple to multiple G protein isoforms.

In addition to the GnRH receptor expressed in GnRH neurons (28), and in other tissues and expression systems (29–32), neurokinin receptor subtypes (NK1 and NK2) exemplify the ability of certain GPCRs to couple to more than one G protein. Similar to the GnRH receptor, agonist activation of both NK receptors elicits a biphasic response with sequential increases in intracellular calcium and cAMP levels (33, 34). However, the cAMP response can be eliminated by point mutations in the extracellular amino-terminal domain of the receptor (35), suggesting that different receptor conformations could result in distinct activation states that have differential ligand affinities, associated with differential coupling to individual G proteins (36). Similarly, mutation of specific residues in the first intracellular loop of the GnRH receptor that are not essential for activation of the phosphoinositide signaling pathway uncouples the receptor from AC signaling (37).

GnRH receptors expressed in native and immortalized GnRH neurons activate diverse signaling pathways by coupling to at least three G proteins. Such coupling is time and dose dependent, and switches between G<sub>q</sub>, G<sub>s</sub>, and G<sub>i/o</sub> according to the agonist concentration (28). These findings suggest that an agonist concentration-dependent switch in coupling of the GnRH receptor between specific G proteins modulates neuronal Ca<sup>2+</sup> signaling via G<sub>s</sub>-cAMP-stimulatory and G<sub>i</sub>-cAMP-inhibitory mechanisms. Activation

of G<sub>i</sub> could also inhibit GnRH neuronal function and episodic secretion by regulating membrane ion currents, probably through activation of G protein-regulated inwardly rectifying potassium channels (38, 39).

In contrast to the single GnRH receptor, RT-PCR analysis of RNA isolated from cultured GT1–7 neurons revealed the expression of mRNAs encoding three specific 5-HT receptors, including the G<sub>i</sub>-coupled 5-HT<sub>1A</sub>, G<sub>q</sub>-coupled 5-HT<sub>2C</sub>, and G<sub>s</sub>-coupled 5-HT<sub>4</sub> and 5-HT<sub>7</sub> receptors. Treatment of native hypothalamic neurons with 5-HT, the endogenous nonselective 5-HT receptor agonist, caused both stimulation and inhibition of AP firing consistent with time- and dose-dependent activation of multiple 5-HT receptor subtypes. Treatment of GT1–7 neurons with 5-HT likewise activates the InsP<sub>3</sub>/Ca<sup>2+</sup> signaling pathway and exerts dose- and time-dependent stimulatory and inhibitory actions on cAMP production and GnRH release in GT1–7 cells. Such changes in InsP<sub>3</sub>/Ca<sup>2+</sup> signaling, cAMP production, and GnRH release were similar to those elicited by activation of the neuronal GnRH receptor (21, 22, 28). These findings indicate that the actions of 5-HT are mediated by activation of multiple signaling pathways, which can account for its previously observed diverse actions on GnRH release (12, 24, 26). Earlier reports on the actions of 5-HT on gonadotropin release indicate that it also has a dual effect on this response that is dependent on estradiol concentration (40), the receptor subtype (41, 42), and interactions with the opiate and adrenergic systems (43).

In addition to its role in the regulation of GnRH release from hypothalamic neurons, 5-HT is an important factor in the early development of the GnRH neuronal system. In Tg8 mice lacking the gene encoding monoamine oxidase A, the number of GnRH neurons in the forebrain was significantly reduced. This suggests that an excess of 5-HT inhibits the proliferation of GnRH neuronal precursor cells and stimulates GnRH neuronal migration to their final location in the septo-preoptic region (44, 45).

A large family of 5-HT<sub>1</sub> receptors is negatively coupled to AC via G<sub>i</sub> proteins. In addition to inhibiting AC, 5-HT<sub>1A</sub> receptors are directly coupled to voltage-sensitive K<sup>+</sup> channels via a G<sub>i</sub>-coupled protein and therefore are not solely dependent on second messenger signaling (46–48). RT-PCR analysis of total RNA isolated from cultured GT1–7 neurons also revealed the expression of 5-HT<sub>1A</sub> G<sub>i</sub>-coupled receptors. Quantitative real-time RT-PCR analysis showed that the expression level of 5-HT<sub>1A</sub> receptors was similar to that of 5-HT<sub>2C</sub> receptors. Treatment of identified hypothalamic GnRH neurons with a 5-HT<sub>1A</sub> receptor agonist (PAPP) caused pronounced inhibition of spontaneous AP firing. This effect was reversible, and spontaneous firing of APs recovered during washout of the 5-HT<sub>1A</sub> agonist. The inhibitory action of 5-HT<sub>1A</sub> receptor activation on spontaneous AP firing in hypothalamic GnRH neurons could be related to the release of  $\beta\gamma$ -subunits from G<sub>i</sub> (or G<sub>o</sub>), with consequent actions on

plasma membrane ion channels (49). Such effects could include both inhibition of voltage-dependent calcium channels (50) and activation of inwardly rectifying potassium channels (51, 52).

Treatment of GT1–7 neurons with the selective 5-HT<sub>1A</sub> receptor agonist, 2-MPP, activated the G<sub>i</sub>-mediated AC/cAMP-inhibitory signaling pathway. This response was prevented by PTX, consistent with coupling to an inhibitory G<sub>i</sub> protein. The resulting decrease in cAMP production was associated with marked inhibition of pulsatile GnRH release. In cultured hypothalamic cells and GT1–7 neurons, such inhibition of pulsatile GnRH release was also observed during activation of G<sub>i</sub>-coupled LH receptors (18), M<sub>2</sub> muscarinic receptors (53), GnRH receptors (28), and estrogen receptors (20). It is evident from our data, and studies by others, that the convergence of signaling from G<sub>i/o</sub>-coupled receptors expressed in native and GT1–7 neurons to the AC/cAMP-inhibitory signaling pathway decreases membrane excitability, reduces the rate of AP firing, and inhibits pulsatile GnRH release. In addition to this process, studies on the role of 5-HT-liberated Gβγ-subunits in synaptic transmission have revealed another inhibitory action of Gβγ on neurosecretion that is distal to Ca<sup>2+</sup> entry and cAMP signaling, and acts directly on the exocytotic fusion machinery (54). This mechanism involves binding of βγ-subunits to the C terminus of SNAP25 and interference with the Ca<sup>2+</sup>-induced soluble N-ethylmaleimide-sensitive factor attachment protein receptor machinery for vesicle fusion and secretory granule exocytosis (55, 56).

The G<sub>q</sub>-coupled 5-HT<sub>2C</sub> receptors expressed in GT1–7 neurons were identified by quantitative RT-PCR as the most abundant of the 5-HT receptors in these cells. In electrophysiological studies, activation of 5-HT<sub>2</sub> receptors in identified embryonic d 18 (E-18) hypothalamic GnRH neurons with the specific receptor agonist, α-methyl 5-HT, significantly increased the frequency of AP firing. This was associated with membrane depolarization, consistent with data obtained during analysis of 5-HT<sub>2C</sub> receptor function in other tissues and expression systems (57). The functional correlates of PLC activation by 5-HT<sub>2</sub> receptors are multiple. An increase in intracellular calcium concentration that induces a rapid Cl<sup>−</sup> current through a Ca<sup>2+</sup>-dependent chloride channel has been characterized for the three members of the 5-HT<sub>2</sub> receptor family and appears to be mediated through the PLC/InsP<sub>3</sub> pathway (58–60). 5-HT<sub>2A</sub> receptor activation also induces the closing of a K<sup>+</sup> channel, leading to depolarization of the cell. In *Xenopus* oocytes coexpressing 5-HT<sub>2C</sub> receptors and a brain-derived K<sup>+</sup> channel, the suppression of K<sup>+</sup> conductance by 5-HT involves a calcium/calmodulin-activated phosphatase, which has been postulated to dephosphorylate the K<sup>+</sup> channel, leading to its closing. Recovery from such suppression may be due to the action of a protein kinase, because it was prevented by the kinase inhibitor H-7 (61).

Members of the 5-HT<sub>2</sub> receptor family are primarily coupled to PLC, and selective activation of these receptors in GT1–7 neurons increased InsP<sub>2+3</sub> production in a dose-dependent manner. This response was pertussis toxin insensitive and was blocked by ketanserin, indicating the involvement of a G<sub>q</sub>-type protein. GnRH release was also affected by selective activation of 5-HT<sub>2C</sub> receptors, which increased both the peak amplitude and the interpulse interval. Similar increases in GnRH pulse amplitude were observed during activation of M<sub>1</sub> muscarinic receptors and activation of GnRH receptors expressed in GT1–7 neurons (28, 53). Thus, increased membrane excitability and the convergence of signaling from G<sub>q</sub>-coupled receptors expressed in GT1–7 cells to common effectors could regulate PLC/InsP<sub>3</sub>/Ca<sup>2+</sup> signaling and increase GnRH peak amplitude.

Stimulation of adenylate cyclase was the first signal transduction pathway to be linked to 5-HT receptors (62). Analysis of the expression of 5-HT<sub>4</sub> receptors in cultured GT1–7 neurons by real-time RT-PCR showed their level of expression to be less than that of 5-HT<sub>2C</sub> receptors. Examination of the electrophysiological properties of G<sub>s</sub>-coupled 5-HT<sub>4</sub> receptors in E-18 hypothalamic GnRH neurons revealed that their spontaneous electrical activity increased during selective agonist treatment with (1*s*,8*s*)-*n*-[(hexahydro-1H-pyrrolizin-1-yl)methyl]-4-amino-5-chloro-2-methoxybenzamide. This increase in AP firing was associated with increased bursting activity and the appearance of lower-amplitude broad APs. This effect has been attributed to cAMP, based on the ability of 8-bromo cAMP and forskolin, a direct activator of adenylate cyclase, to mimic the actions of 5-HT (63). Depression of K<sup>+</sup> current may lead to depolarization, calcium influx, and subsequent enhancement of GnRH release (8).

Selective activation of 5-HT<sub>4</sub> receptors in GT1–7 neurons increased cAMP production in a dose-dependent manner and caused a robust and sustained increase in GnRH secretion during perfusion studies. Similar increases in GnRH release were observed during activation of β<sub>1</sub>-adrenergic receptors (64) and application of 8-bromo cAMP and forskolin (18, 65). Our observations, and those of others, indicate that cAMP signaling from G<sub>s</sub>-coupled receptors expressed in GT1–7 cells increases membrane excitability and causes prominent and sustained increases in GnRH secretion (8, 66).

In summary, the marked inhibitory effect of G<sub>i</sub>-coupled 5-HT<sub>1A</sub> receptors on spontaneous AP firing, cAMP production, and inhibition of pulsatile GnRH secretion suggests their involvement in negative regulation during pulsatile GnRH release. Activation of 5-HT<sub>2</sub> G<sub>q</sub>-coupled receptors triggers the PLC/InsP<sub>3</sub>/Ca<sup>2+</sup> signaling pathway and promotes spike-like increases in GnRH release. Selective targeting of 5-HT<sub>4</sub> receptors activates the G<sub>s</sub>-AC-cAMP signaling pathway, increases cAMP production and AP firing frequency, and stimulates basal GnRH release. In addi-

tion to the GnRH receptor, native and immortalized GnRH neurons express several 5-HT and other G protein-coupled receptors. These observations indicate that the convergence of signaling from specific GPCRs to common effector systems provides a powerful mechanism for the control of pulsatile GnRH release.

## MATERIALS AND METHODS

### Tissue and Cell Culture

Hypothalamic tissue was removed from fetuses of 17-d pregnant Sprague Dawley rats (Charles River Laboratories, Wilmington, MA). The borders of the excised hypothalami were delineated by the anterior margin of the optic chiasm, the posterior margin of the mammillary bodies, and laterally by the hypothalamic sulci. After dissection, hypothalami were placed in ice-cold dissociation buffer containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM HEPES, and 100 mg/liter gentamicin, pH 7.4. The tissues were washed and then incubated in a sterile flask with dissociation buffer supplemented with 0.2% collagenase, 0.4% BSA, 0.2% glucose, and 0.05% DNase I. After 60 min incubation in a 37°C water bath with shaking at 60 cycles/min, the tissue was gently triturated by repeated aspiration into a smooth-tipped Pasteur pipette. Incubation was continued for another 30 min, after which the tissue was again dispersed. The cell suspension was passed through sterile mesh (200  $\mu$ m) into a 50-ml tube, sedimented by centrifugation for 10 min at 200  $\times$  g, and washed once in dissociation buffer and once in culture medium consisting of 500 ml DMEM containing 0.584 g/liter L-glutamate and 4.5 g/liter glucose, mixed with 500 ml F-12 medium containing 0.146 g/liter L-glutamine, 1.8 g/liter glucose, 100  $\mu$ g/ml gentamicin, 2.5 g/liter sodium bicarbonate, and 10% heat-inactivated fetal calf serum. Each dispersed hypothalamus yielded about  $1.5 \times 10^6$  cells. Immortalized GnRH neurons (GT1-7 cells) were provided by Dr. Richard Weiner (University of California at San Francisco) and were cultured under the same conditions as primary hypothalamic cells.

### Cell Perfusion Procedure and Hormone Measurement

Bead-attached GT1-7 cells were perfused at a flow rate of 0.15 ml/min at 37°C, and fractions collected at 5-min intervals were stored at –20°C before RIA of their GnRH content. GnRH was measured using <sup>125</sup>I-labeled GnRH (Amersham Biosciences Corp., Piscataway, NJ), GnRH standards (Peninsula Laboratories, Belmont, CA), and primary antibody donated by Dr. V. D. Ramirez (University of Illinois, Urbana, IL). The intra- and interassay coefficients of variation at 50% binding in standard samples (15 pg/ml) were 5% and 7%, respectively. The sensitivity of the assay, defined as twice the SD at zero dose, was 0.2 pg/tube ( $n = 6$ ). In previous studies, pulsatile GnRH secretion and its regulation have been found to be identical in GT1-7 cells and cultured hypothalamic neurons.

### cAMP Production

For studies on cAMP release, GnRH-producing cells were stimulated in serum-free medium (1:1 DMEM/F-12) containing 0.1% BSA, 30 mg/liter bacitracin, and 1 mM 3-isobutyl-1-methylxanthine. RIA of cAMP was performed as previously described, using a specific cAMP antiserum at a titer of 1:5000 (67). The intraassay coefficient of variation of the assay was 4% at 50% displacement.

### Inositol Phosphate Production

Cells were labeled for 24 h in inositol-free medium containing 20 mCi/ml [<sup>3</sup>H]inositol as described previously (68), and then washed with inositol-free M199 medium and stimulated at 37°C in the presence of 10 mM LiCl. Inositol phosphates were fractionated by anion exchange chromatography, and the InsP<sub>2+3</sub> fractions eluted with 1 M ammonium formate in 0.1 M formic acid (3 ml/wash) were analyzed by liquid scintillation b-spectrometry.

### Whole-Cell Recording of GnRH Neurons

For whole-cell recording, hypothalamic cells were cultured on collagen-coated cover slips and continuously perfused with artificial extracellular solution at a rate of 0.6 ml/min. The extracellular solution contained (in mM): 140 NaCl, 5 KCl, 10 HEPES, 10 D-glucose, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>. pH was adjusted to 7.4 with NaOH. The cells were viewed under an inverted Olympus IX70 microscope with a  $\times 40$ , long working distance objective (Olympus Corp., Lake Success, NY). All recordings were done at room temperature (23–25°C). Patch pipettes (3–5 M $\Omega$ ) were pulled from thick-wall borosilicate capillary glass (1.5-mm outer diameter and 0.86-mm inner diameter, WPI, Inc., Sarasota, FL) on a Flaming/Brown puller model P-87 (Sutter Instruments Co., Novato, CA). The pipette solution was prepared containing (in millimolar concentration): 70 KCl, 70 potassium gluconate, 0.1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPES, 2 ATP potassium salt (K<sub>2</sub>ATP), 0.1 Na<sub>2</sub>GTP, 5 EGTA, with pH adjusted to 7.2 with KOH. An Ag/AgCl pellet was used as the reference electrode. Spontaneous activities were recorded under I-clamp mode with a Multi-Clamp700A amplifier (Axon Instruments, Foster City, CA), filtered at 2 kHz, and digitized at 10 kHz through Digidata 1320A (Axon Instruments). Acquisition and subsequent analysis of the experimental data were performed using Clampex 9.0 software (Axon Instruments). Traces and voltage-current curves were plotted using “Origin 7” computer software (MicroCal Software, Northampton, MA). After recordings, the cytoplasmic contents of the recorded neuron were harvested under visual control, and single-cell RT-PCR was used to identify the presence of GnRH transcripts as previously reported (27, 69). Hypothalamic cells that did not show typical GnRH neuronal morphology were used as controls. Firing of APs in identified cultured E-18 hypothalamic GnRH neurons was obtained from single isolated GnRH neurons to eliminate the influence of electrical and synaptic coupling between cells.

### RT-PCR Analysis of 5-HT Receptor Subtype

Total RNA was extracted from GT1-7 cells using Absolutely RNA RT-PCR Miniprep Kits (Stratagene, La Jolla, CA). RNA was digested with DNase in a low-salt buffer to remove any remaining DNA. Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Briefly, using 5  $\mu$ g of total RNA as a template, first-strand cDNA was made using 500 ng of oligo(dT)12–18 and 1 ml of 10 mM deoxynucleotide triphosphate Mix (Invitrogen) in a 13- $\mu$ l reaction volume. After heat denaturing at 65°C for 5 min, and addition of 4  $\mu$ l of 5 $\times$  First Strand Buffer, 1  $\mu$ l of 0.1 M dithiothreitol, 1  $\mu$ l of RNase OUT recombinant RNase inhibitor (Invitrogen), and 200 U of SuperScript III Reverse Transcriptase, reverse transcription was performed at 55°C for 50 min. RNA complementary to the cDNA was removed by addition of 1  $\mu$ l of *E. coli* RNase H and incubation at 37°C for 20 min. An 0.5- $\mu$ l aliquot of cDNA was used as template. Primers used were 5'-G[nucleotide (nt) 442] CATTCTTTT-TCCCTCCCTCC (nt 464)-3' (sense) and 5'-T(nt 809) GAC-CCAGAGTCCACTTGTTGAG (nt 787)-3' (antisense) for 5-HT1A receptor; 5'-T(nt 2345) CTCCCTTCCTCCGTAT-TCCC (nt 2366)-3' (sense) and 5'-T(nt 2796) GGCATCCTTC-CACTTCTGTAGTC (nt 2773)-3' (antisense) for 5-HT2C re-

ceptor; 5'-T(nt 25) GAGTTCCAACGAGGGTTTCAG (nt 46)-3' (sense) and 5'-T (nt 422) AATGCGATGCGTAGAGGGG (nt 403)-3' (antisense) for 5-HT<sub>4</sub> receptor; and 5'-G(nt 1137) CTGCCGTTTTCTCTTGTG (nt 1157)-3' (sense) and 5'-C(nt 1513) AATGGTTTCGTTGTTTCCCC (nt 1494)-3' (antisense) for 5-HT<sub>7</sub> receptor; and 5'-A(nt 152) ACGACCCCTTCATTGAC (nt 1169)-3' (sense) and 5'-T(nt 342) CCACGACATACTCAGCAC (nt 324)-3' (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expected sizes of 5HT<sub>1</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>4</sub>, 5HT<sub>7</sub>, and GAPDH were 321, 347, 315, 431, and 191 bp, respectively. PCR conditions were: denaturing at 94 C for 2 min, followed by 30 cycles of denaturing at 94 C for 30 sec, annealing at 55 C for 30 sec, and extension at 72 C for 60 sec. PCR products were analyzed by electrophoresis using 2% agarose gels.

### Real-Time RT-PCR Analysis of 5-HT Receptor Expression

mRNA of 5HT receptors were quantified by real-time RT-PCR performed in a Light Cycler (Roche, Branchburg, NJ) using SYBR Green I as a double-strand DNA-specific binding dye according to the manufacturer's instructions, continuously monitoring the cycle-by-cycle accumulation of each fluorescently labeled PCR product. Amplifications were carried out using 1 U Platinum Taq DNA polymerase (Invitrogen), 0.5  $\mu$ M of each primer, 3 mM MgCl<sub>2</sub>, 10xPlatinum Taq DNA polymerase buffer (200  $\mu$ M/liter Tris-HCL, pH 8.4; 500  $\mu$ M/liter KCl), 200  $\mu$ M deoxynucleotide triphosphate, 1 mg/ml BSA, 1  $\mu$ l 1:2000 dilution of SYBR Green I nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME), and 2  $\mu$ l 1:5 dilution of cDNA in a total volume of 20  $\mu$ l. The real-time PCR conditions were preheat denaturation at 95 C for 5 min, annealing at 59 C for 10 sec, and extension at 72 C for 12 sec; cycle number 45. SYBR Green I fluorescence was detected at 72 C at the end of each cycle to monitor the amount of PCR product formed. A melting curve analysis of the amplification products was performed at the end of the PCR run by rapidly increasing the temperature to 95 C, followed by immediate cooling to 65 C for 15 sec, after which the temperature was gradually increased to 95 C at a rate of 0.1 C per second with continuous measurement of fluorescence to confirm amplification of specific transcripts. The melting temperature profile for 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>4</sub>, 5-HT<sub>7</sub> receptors and GAPDH demonstrated single peaks at 85.5 C, 86 C, 90 C, 86 C, and 89 C, respectively.

External cDNA standards for 5-HT receptors and GAPDH were produced by inserting PCR products, which were generated using the same primers used for RT-PCR and GT1-7 cell cDNA as a template, into the pCR2.1 vector using the TOPO TA Cloning Kit (Invitrogen). Vector constructs were used to transform DH5 $\alpha$ , and plasmid DNA was prepared by Wizard plus Minipreps DNA purification System (Promega Corp., Madison WI). The inserts of control vectors for 5-HT receptors were verified by sequencing. The concentration of standard was determined by measuring the OD<sub>260</sub>, and the copy number was calculated.

### Materials

Oligonucleotides were obtained from Gene Probe Technologies (Gaithersburg MD). Absolutely RNA RT-PCR Miniprep Kit was purchased from Stratagene (La Jolla, CA). SuperScript III RNase H<sup>-</sup> radical extender. Reverse Transcriptase, Platinum Taq DNA polymerase, pCR2.1 vector, and TOPO TA cloning kit were purchased from Invitrogen. Wizard plus Minipreps DNA purification system was purchased from Promega Corp.

5-HT, the nonselective endogenous 5-HT receptor agonist, was purchased from Sigma-Aldrich (St. Louis, MO). Selective 5-HT<sub>1</sub> receptor agonist analogs, 2-MPP and PAPP, were purchased from Sigma-Aldrich. Selective 5-HT<sub>2</sub> recep-

tor agonist analog  $\alpha$ -methyl-5-HT maleate ( $\alpha$ -methylserotonin maleate), and ketanserin tartrate-selective 5-HT<sub>2</sub> antagonist were purchased from Sigma-Aldrich. The (1s,8s)-n-[(hexahydro-1H-pyrrolizin-1-yl)methyl]-4-amino-5-chloro-2-methoxy-benzamide-selective 5-HT<sub>4</sub> agonist was a gift from Searle Pharmaceuticals (High Wycombe, UK).

### Data Analysis

GnRH pulses were identified and their parameters determined by computerized cluster analysis (70). Individual point sds were calculated using a power function variance model from the experimental duplicates. A 2  $\times$  2 cluster configuration and a *t* statistic of 2 for the upstroke and downstroke were used to maintain false-positive and false-negative error rates below 10%. The pulse parameters were analyzed by ANOVA and results expressed as mean  $\pm$  SEM. Statistical comparisons were performed using the Kruskal-Wallis test followed by the Mann-Whitney *U* test.

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